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Title: **METHODS AND COMPOSITIONS FOR THE TREATMENT OF FIBROTIC CONDITIONS & IMPAIRED LUNG FUNCTION & TO ENHANCE LYMPHOCYTE PRODUCTION**

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METHODS AND COMPOSITIONS FOR THE TREATMENT OF FIBROTIC CONDITIONS & IMPAIRED LUNG FUNCTION & TO ENHANCE LYMPHOCYTE PRODUCTION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of the following applications: U.S. Application Serial No. 09/120,264, filed July 21, 1998, which is a continuation-in-part of U.S. Application Serial No. 09/087,210, filed May 28, 1998, which is a continuation-in-part of U.S. Application Serial No. 08/864,357, filed May 28, 1997. The disclosures of each of the aforementioned applications are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to the use of human uteroglobin or recombinant human uteroglobin in the treatment of fibrotic conditions, to increase lymphocyte production *in vivo*, and to improve and/or normalize lung function, pulmonary compliance, blood oxygenation, and blood pH. Novel physiological roles and therapeutic targets for uteroglobin have been identified. Specifically, the invention first provides a method of inhibiting cell adhesion to fibronectin by administering human uteroglobin or recombinant human uteroglobin. The invention also provides a method of increasing lymphocyte production *in vivo* by administering human uteroglobin or recombinant human uteroglobin. Finally, the invention provides a method of improving lung function by administering human uteroglobin or recombinant human uteroglobin.

Documents cited in this application relate to the state-of-the-art to which this invention pertains. The disclosures of each of these references are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Uteroglobin (also known as UG, CC10, CC16, CC17, urine protein-1, P-1, progesterone binding protein, PCB-binding protein, Clara cell secretory protein (CCSP), blastokinin, retinol-binding protein, phospholipid-binding protein, and alpha2-microglobulin) is a highly conserved mammalian protein that is primarily produced by the pulmonary epithelia. It is present in the mucosal fluid of the respiratory tract, circulates in the blood, and is excreted in the urine. Uteroglobin is a small globular homodimeric protein that consists of two identical seventy amino

acid peptides that complex in an anti-parallel orientation. It has a molecular weight of 15.8 kDa, but it migrates in electrophoretic gels at a size corresponding to 10 kDa. Two disulfide bonds spontaneously form to covalently link the monomers as a dimer. Human uteroglobin is abundant in the adult human lung, and comprises up to about 7% of the total soluble protein. However, its expression is not fully activated in the developing human fetus until late in gestation. Consequently, the extracellular lung fluids of pre-term infants contain far less human uteroglobin than those of adults. Uteroglobin is also expressed by the pancreas.

Amino acid analysis of purified human uteroglobin reveals that it is structurally similar but not identical to other uteroglobin-like proteins, e.g. rabbit uteroglobin; 39 of 70 amino acids are identical between human and rabbit uteroglobin. The uteroglobin-like proteins, including human uteroglobin, rat uteroglobin, mouse uteroglobin, and rabbit uteroglobin, exhibit species-specific and tissue-specific antigenic differences, as well as differences in their tissue distribution and biochemical activities *in vitro*. Uteroglobin-like proteins have been described in many different contexts with regard to tissue and species of origin, including rat lung, human urine, sputum, blood components, rabbit uterus, rat and human prostate, and human lung.

The absence of structural identity among uteroglobin-like proteins makes it impossible to predict whether a protein will possess *in vivo* therapeutic function in humans based on *in vitro* or other activity exhibited by a structurally related protein. For example, human uteroglobin binds less than 5% of the amount of progesterone as rabbit uteroglobin binds in the same assay. In addition, human uteroglobin has a lower isoelectric point (4.6) than rabbit uteroglobin (5.4).

Uteroglobin is known to inhibit the enzymatic activity of secretory (soluble) phospholipases A2 ($sPLA_2$s) which hydrolyze phospholipids, sometimes releasing arachidonic acid in the process. Arachidonic acid is a precursor for several pro-inflammatory and anti-inflammatory eicosanoids. The role of uteroglobin as an anti-inflammatory agent *in vivo* was confirmed by the discovery of an inflammatory phenotype in the organs of a transgenic uteroglobin knockout mouse (USSN 08/864,357). The renal fibrotic phenotype of the uteroglobin knockout mouse also led to the discovery that uteroglobin forms a complex with fibronectin, preventing fibronectin aggregation and deposition *in vivo* (USSN 08/864,357). In addition, it was found that uteroglobin prevents the formation of a complex between fibronectin and IgA. However, this animal exhibits no pulmonary phenotype.

The renal fibrotic phenotype of the uteroglobin knockout mouse first disclosed in USSN

08/864,357 led to the discovery that uteroglobin may play a significant role in controlling fibronectin aggregation and deposition. Fibronectin is a 200 kDa glycoprotein which exists in several different forms and is secreted by different tissues. Fibronectin is an essential protein and targeted disruption of the fibronectin gene in mice showed that it has a central role in embryogenesis. Fibronectin also plays a key role in inflammation, cell adhesion, tissue repair and fibrosis, and is deposited at the site of injury. Plasma fibronectin is secreted by the liver and circulates in the plasma. In the lung, cellular fibronectin is secreted upon inflammation and injury. Both types of fibronectin are chemotactic factors for inflammatory cells and fibroblasts. Large numbers of inflammatory cells and fibroblasts infiltrate the lung during inflammatory episodes, which can lead to pulmonary fibrosis and ultimately death. Elevated levels of fibronectin have been detected in human clinical conditions such as neonatal respiratory distress syndrome and bronchopulmonary disease of the lung, and glomerular nephropathy of the kidney.

However, the physiological role of uteroglobin remains a source of controversy in the art. Stripp *et al.* (1996) also generated a uteroglobin knockout mouse in which the expression of uteroglobin was eliminated. The mouse has Clara cells which exhibit odd intracellular structures in place of uteroglobin secretion granules, but there is no other life-threatening phenotype. This knockout mouse also showed no evidence of renal, pancreatic, or reproductive abnormality. These results are completely at odds with the observations made from the uteroglobin knockout mouse described in USSN 08/864,357. This mouse does, however, exhibit exacerbated pulmonary inflammation when challenged with pulmonary insult.

Leyton et al. (1994) reported the anti-metastatic properties of uteroglobin which were attributed to its inhibition of the release of arachidonic acid by tumor cells. (See also U.S. Patent No. 5,696,092 to Patierno et al.) Kundu et al. (1996) continued this work with the observation of inhibition of extracellular matrix invasiveness by a variety of tumor cell types. Extracellular matrix invasion correlated with the presence of a 190 kDa uteroglobin binding protein in responsive cell types. The extracellular matrix invasion activity of cells lacking this protein could not be inhibited by uteroglobin.

New investigations into the therapeutic properties of uteroglobin in non-murine animal models has led to the discovery of novel mechanisms of action *in vivo* that are distinct from the effects of uteroglobin on inflammation and fibrosis previously observed by skilled artisans in the field.

OBJECT OF THE INVENTION

It is an object of the present invention to provide a method of improving and/or normalizing lung function, pulmonary compliance, blood oxygenation, and/or blood pH by administering an effective amount of human uteroglobin or recombinant human uteroglobin.

It is also an object of the invention to provide a composition consisting of an amount of human uteroglobin or recombinant human uteroglobin sufficient to improve and/or normalize lung function, pulmonary compliance, blood oxygenation, and/or blood pH. Such a composition should include a pharmaceutically acceptable carrier or diluent, and the composition should preferably consist of oxidized dimeric recombinant human uteroglobin.

Further, it is an object of the invention to provide a method of increasing lymphocyte production *in vivo* by administering an amount of human uteroglobin or recombinant human uteroglobin sufficient to increase lymphocyte production and/or decrease polymorphonuclear leukocyte proliferation. Preferably, the concentration of effector lymphocytes and/or cytotoxic T cells is increased by the administration of uteroglobin. Moreover, it is an object of the invention to administer uteroglobin to increase lymphocyte production and/or decrease polymorphonuclear leukocyte proliferation in patients suffering from an autoimmune disease or allergy.

It is an additional object of the invention to provide a composition consisting of an amount of human uteroglobin or recombinant human uteroglobin sufficient to increase lymphocyte production and/or decrease polymorphonuclear leukocyte proliferation, together with a pharmaceutically acceptable carrier or diluent.

Still further, it is an object of the present invention to provide a method of inhibiting cellular adhesion to fibronectin by administering an amount of human uteroglobin or recombinant human uteroglobin sufficient to inhibit cellular adhesion to fibronectin *in vivo*. It is a further object of the invention to inhibit inflammatory cell and fibroblast migration on fibronectin already deposited *in vivo*, and to inhibit the interaction between a cell and an extracellular matrix protein and/or membrane bound protein.

It is a final object of the present invention to provide a composition consisting of an amount of human uteroglobin or recombinant human uteroglobin sufficient to inhibit cellular adhesion to fibronectin *in vivo*. Such compositions should consist of a pharmaceutically acceptable carrier or diluent.

SUMMARY OF THE INVENTION

It has now been found that uteroglobin plays a central physiological role in fibronectin deposition, lymphocyte production, smooth muscle function, and lung function *in vivo*.

In a first experiment it was found that the administration of uteroglobin to neonatal lambs delivered by caesarian section, an accepted model for surfactant-dependent neonatal respiratory distress syndrome in humans, led to improved and/or normalized blood oxygenation and pH. These effects are indicative of improved lung function. This observation, discussed in more detail below, is the first observation of the direct effect of uteroglobin on lung tissue and the first indication that uteroglobin may be used to improve and/or normalize lung function.

Further, in a second experiment using newborn piglets, it was found that the administration of recombinant human uteroglobin increased pulmonary compliance. The newborn piglet is an excellent model for neonatal lung injury mediated by oxygen toxicity arising from the use of positive pressure ventilation and elevated oxygen delivery in respiratory distress syndrome rescue. This significant observation was the first indication that uteroglobin may be used to increase pulmonary compliance. This effect was independent of any effects of uteroglobin on surfactant function. These data show that uteroglobin may be used to treat patients suffering from reduced pulmonary compliance as a result of a pulmonary challenge or insult resulting from exposure to non-atmospheric gases, inhaled chemicals, pollutants, irritants, pollens, allergens, particulate matter, and airborne infectious agents. It was also found that a single dose of recombinant human uteroglobin to newborn piglets significantly increased lymphocyte proliferation and decreased polymorphonuclear leukocyte proliferation. The increase in lymphocyte proliferation was significant, up to 2.5 fold, and the decrease in polymorphonuclear leukocyte proliferation of up to 2.3 fold persisted for a period exceeding one month.

Finally, using two new assay formats designed to specifically detect uteroglobin-fibronectin binding, it was found that recombinant human uteroglobin binds to portions of fibronectin that are important in cell adhesion and not known to be relevant to fibrillogenesis. Fibronectin consists of eight type I domains in the N-terminal third of the protomer, three type I domains at the C-terminus, two type II domains clustered in the middle of the protomer, and 15-17 type III domains, depending on the tissue of origin. One or more of the type III domains have been implicated in cell adhesion, fibronectin-fibronectin interactions, and deposition *in vitro*. Using two commercially available chymotryptic fragments of fibronectin, each containing type III domains involved in

fibronectin-dependent cell adhesion and/or polymerization, and a recombinant fragment of fibronectin, termed "superfibronectin" (so named because of its ability to promote fibronectin-fibronectin interactions, polymerization, deposition, and cell adhesion *in vitro*), the interaction between uteroglobin and the various regions of fibronectin was examined.

A clear dose-response relationship in binding between recombinant human uteroglobin and "superfibronectin" was observed. This indicates that recombinant human uteroglobin binds to the type III domain of fibronectin which is represented by "superfibronectin". Further, it was also found that recombinant human fibronectin binds to more than one type III domain of fibronectin because a dose-response relationship was observed for binding between uteroglobin and a chymotryptic fragment that does not contain the "superfibronectin" domain. Still further, because fibronectin type III domains are present in nearly all components of the extracellular matrix, e.g., laminin, collagens, vitronectin, and fibrin, as well as in numerous membrane bound proteins, e.g., adhesion molecules, integrins, and receptors, these domains may play a central role in cell-cell and cell-extracellular matrix interactions. The observation that uteroglobin interacts with these domains shows that it may be used to mediate these interactions and physiological conditions affected by such interactions.

Therefore, according to one aspect of the present invention, the invention provides a method of treatment including improving and/or normalizing lung function in a patient in need of such treatment, wherein the method consists of administering an amount of uteroglobin effective to improve and/or normalize lung function relative to that observed in the absence of such treatment. In a preferred embodiment, the uteroglobin is recombinant human uteroglobin, and the preferred dosage range is 10 ng/kg - 25 mg/kg.

According to a further aspect, the invention provides a method of improving and/or normalizing pulmonary compliance in a patient in need of such treatment, wherein the method consists of administering an amount of uteroglobin to the patient sufficient to improve and/or normalize pulmonary compliance relative to that observed in the absence of such treatment. In a preferred embodiment, the uteroglobin is recombinant human uteroglobin, and the preferred dosage range is 10 ng/kg - 25 mg/kg. The reduced pulmonary compliance may have resulted from pulmonary challenge or insult resulting from exposure to non-atmospheric gases, inhaled chemicals, pollutants, irritants, inhaled pollens, allergens, particulate matter, and airborne infectious agents.

An additional aspect of the invention provides a method of treating a patient suffering from reduced blood oxygenation and/or blood pH, wherein the method includes administering an amount of uteroglobin effective to improve and/or normalize blood oxygenation and/or blood pH relative to that observed in the absence of such treatment. In a preferred embodiment, the uteroglobin is recombinant human uteroglobin, and the preferred dosage range is 10 ng/kg - 25 mg/kg.

Another aspect of the invention provides compositions consisting of uteroglobin effective for the improvement and/or normalization of lung function, pulmonary compliance, blood oxygenation, and/or blood pH. Such compositions preferably contain a dosage of 10 ng - 500 mg, in combination with a pharmaceutically acceptable carrier or diluent, wherein the amount of uteroglobin contained in the composition is commensurate with the administration of 10 ng/kg - 25 mg/kg by the method of the present invention. In a preferred embodiment, the uteroglobin is recombinant human uteroglobin.

According to a further aspect, the invention provides a method of increasing lymphocyte production in a patient in need of such treatment, wherein the method includes administering an amount of uteroglobin sufficient to increase lymphocyte production in the patient relative to that observed in the absence of such treatment. In a preferred embodiment, recombinant human uteroglobin is used at a dosage range of 1 ng/kg - 100 mg/kg. Preferably, the method increases the production of effector lymphocytes and/or cytotoxic T cells. The patient may be suffering from decreased lymphocyte production as a result of an autoimmune disease, such as acquired immunodeficiency syndrome, or an allergy. In addition, uteroglobin may be used to enhance a lymphocyte response to a vaccine.

In an additional aspect, the invention provides a method of increasing the production of suppressor T cells in a patient in need of such treatment, wherein the method includes administering an amount of uteroglobin sufficient to increase production of suppressor T cells in the patient relative to that observed in the absence of such treatment. In a preferred embodiment, recombinant human uteroglobin is used at a dosage range of 1 ng/kg - 100 mg/kg.

In accordance with an additional aspect, the invention provides a method of enhancing a lymphocyte-mediated response in a patient in need of such treatment, wherein the method includes administering an amount of uteroglobin sufficient to enhance such a response in the patient relative to that observed in the absence of such treatment. In a preferred embodiment, recombinant human uteroglobin is used at a dosage range of 1 ng/kg - 100 mg/kg.

A further aspect of the invention is to provide a method of decreasing the production of polymorphonuclear leukocytes in a patient in need of such treatment, wherein the method includes administering an amount of uteroglobin sufficient to decrease polymorphonuclear leukocyte production in the patient relative to that observed in the absence of such treatment. In a preferred embodiment, recombinant human uteroglobin is used at a dosage range of 1 ng/kg - 100 mg/kg.

An aspect of the invention is to provide compositions including uteroglobin in an amount sufficient to increase production of lymphocytes and/or suppressor T cells, enhance a lymphocyte-mediated response, and/or to decrease the production of polymorphonuclear leukocytes in a patient in need of such treatment. In a preferred embodiment, recombinant human uteroglobin is used in the composition at a dosage range of 1 ng/kg - 100 mg/kg, together with a pharmaceutically acceptable carrier or diluent.

According to an additional aspect, the invention provides a method of treatment including inhibiting fibronectin-dependent cell adhesion to fibronectin in a patient in need of such treatment, wherein the method consists of administering an amount of uteroglobin to the patient sufficient to inhibit fibronectin-dependent cell adhesion to fibronectin relative to that observed in the absence of such treatment. In a preferred embodiment, the uteroglobin is recombinant human uteroglobin, and the preferred dosage range is 8 μg - 3.5 g dose per 70 kg patient. Most preferably, the dosage range is 25 ng/200 μl - 10 μg/200 μl. In an additional preferred embodiment, uteroglobin blocks cell adhesion to type III domains of fibronectin.

An aspect of the invention provides a method of treatment including inhibiting an interaction between fibronectin and cells dependent on fibronectin binding, wherein the method includes administering an amount of uteroglobin effective to inhibit such interactions relative to that observed in the absence of such treatment. In a preferred embodiment, the uteroglobin is recombinant human uteroglobin, and the preferred dosage range is 8 μg - 3.5 g dose per 70 kg patient. Most preferably, the dosage range is 25 ng/200 μl - 10 μg/200 μl.

According to a further aspect, the invention provides a method of inhibiting inflammatory cell and fibroblast migration on fibronectin already deposited *in vivo* in a patient in need of such treatment, wherein the method includes administering an amount of uteroglobin sufficient to inhibit fibronectin-dependent cell adhesion to fibronectin in the patient relative to that observed in the absence of such treatment. In a preferred embodiment, the uteroglobin is recombinant human

uteroglobin, and the preferred dosage range is 8 μg - 3.5 g dose per 70 kg patient. Most preferably, the dosage range is 25 ng/200 μl - 10 μg/200 μl.

In another aspect, the invention provides a method of inhibiting fibronectin-dependent cell adhesion in a patient in need of such treatment, wherein the method includes administering an amount of uteroglobin sufficient to inhibit fibronectin-dependent cell adhesion in the patient relative to that observed in the absence of such treatment.

In accordance with an aspect, the invention provides a method of inhibiting an interaction betweeen a cell and an extracellular matrix protein and/or membrane bound protein in a patient in need of such treatment, wherein the method includes administering an amount of uteroglobin sufficient to inhibit such interactions in the patient relative to that observed in the absence of such treatment. In a preferred embodiment, the uteroglobin is recombinant human uteroglobin, and the preferred dosage range is 8 μg - 3.5 g dose per 70 kg patient. Most preferably, the dosage range is 25 ng/200 μl - 10 μg/200 μl.

A final aspect of the invention is to provide compositions including uteroglobin in an amount sufficient to inhibit fibronectin-dependent cell adhesion to fibronectin, an interaction between fibronectin and cells dependent on fibronectin binding, inflammatory cell and fibroblast migration on fibronectin deposited *in vivo*, fibronectin-dependent cell adhesion, and an interaction between a cell and an extracellular matrix protein and/or membrane bound protein in a patient in need of such treatment. In a preferred embodiment, recombinant human uteroglobin is used in the composition, together with a pharmaceutically acceptable carrier or diluent, and the preferred dosage range is 8 μg - 3.5 g dose per 70 kg patient. Most preferably, the dosage range is 25 ng/200 μl - 10 μg/200 μl.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in more detail, with reference to the accompanying drawings, in which:

Figure 1 shows the standard curve obtained using the uteroglobin (UG) immunoassay described below.

Figure 2 shows the bicarbonate excess (BE) exhibited in pre-term lambs upon intratracheal administration of recombinant human uteroglobin.

Figure 3 shows the decrease in CO_2 exhibited in pre-term lambs upon intratracheal administration of recombinant human uteroglobin.

Figure 4 shows the increase in blood pH exhibited in pre-term lambs upon intratracheal administration of recombinant human uteroglobin.

Figure 5 shows the increase in paO_2/FiO_2 exhibited in pre-term lambs upon intratracheal administration of recombinant human uteroglobin.

Figure 6 shows the concentration of recombinant human uteroglobin (CC10) in serum as a function of time after intratracheal administration of recombinant human uteroglobin to newborn piglets.

Figure 7 shows the pressure-volume relationship observed upon administration of recombinant human uteroglobin to newborn piglets.

Figure 8 shows the mean pressure-volume relationship observed upon administration of recombinant human uteroglobin to newborn piglets ventilated with 100% oxygen.

Figure 9 shows the mean pressure-volume relationships observed for all animals upon administration of recombinant human uteroglobin to newborn piglets ventilated with room air and 100% oxygen.

Figure 10 shows the mean pulmonary compliance among all groups of newborn piglets administered recombinant human uteroglobin.

Figure 11 shows radioactive counts as a function of time for each group of Wistar rats administered recombinant human uteroglobin via intravenous administration.

Figure 12 shows radioactive counts as a function of time for each group of Wistar rats administered recombinant human uteroglobin via intranasal administration.

Figure 13 shows radioactive counts as a function of time for each group of Wistar rats administered recombinant human uteroglobin via stomach gavage.

Figure 14 shows the concentration of recombinant human uteroglobin as a function of time for each group of Wistar rats administered recombinant human uteroglobin via intravenous administration.

Figure 15 shows the concentration of recombinant human uteroglobin as a function of time for each group of Wistar rats administered recombinant human uteroglobin via intranasal administration.

Figure 16 shows the concentration of recombinant human uteroglobin as a function of time

for each group of Wistar rats administered recombinant human uteroglobin via stomach gavage.

Figures 17A-17B are schematic representations of two ELISA-based assay formats for the uteroglobin-fibronectin binding interaction. Format A, shown in Figure 17A represents an assay based on immunodetection, wherein CC10 is uteroglobin and HRP is horse radish peroxidase. Format B, shown in Figure 17B represents a competitive binding assay format in which CC10 is uteroglobin, HRP is horse radish peroxidase, and rhFn is recombinant human fibronectin, and the free uteroglobin in the same competes with the HRP-labeled uteroglobin for binding sites on recombinant human fibronectin.

Figure 18 shows a map of the human fibronectin protomer.

Figure 19 shows the results obtained from binding assays between uteroglobin (CC10) and intact and fragmented fibronectin using format A, wherein hFn is human fibronectin and SuperFn is superfibronectin.

Figures 20A-20B show the dose response curves for uteroglobin binding to fibronectin and its fragments using format A, wherein rhUG is recombinant human uteroglobin, hFn is human fibronectin, and Fn is fibronectin.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Definitions

Native and recombinant human uteroglobin may be used in the present invention. In a preferred embodiment, however, recombinant human uteroglobin is employed in the methods and compositions of the invention. The recombinant form of uteroglobin preferably has substantially the same amino acid sequence as that of the native human uteroglobin protein. An amino acid sequence having "substantially the same" amino acid sequence as that of the native human protein includes recombinant human uteroglobin having at least 75% identity to the native human protein. In a preferred embodiment, recombinant human uteroglobin has at least 85% identity, and in a most preferred embodiment, recombinant human uteroglobin has at least 98% identity to the native uteroglobin. In a further preferred embodiment, oxidized dimeric recombinant human uteroglobin is used in the methods and compositions of the present invention (with respect to the various forms of uteroglobin, reference is made to USSN 09/120,264).

Also included in the method of the present invention is the use of fragments or derivatives of uteroglobin, native or recombinant. A "fragment" of uteroglobin refers to a portion of the native

uteroglobin amino acid sequence having six or more contiguous amino acids of the native protein sequence. The term "derivative" refers to peptide analogs of uteroglobin, including one or more amino acid substitutions and/or the addition of one or more chemical moieties, e.g., acylating agents, sulfonating agents, carboxymethylation of the disulphide bonds, or complexed or chelated metal or salt ions, e.g. Mg^{+2}, Ca^{+2}, or Na^{+1}, with the proviso that the derivative retains the biological activity of the parent molecule. In addition, the present invention also contemplates the use of small molecule mimetics and chemical structural derivatives of uteroglobin.

A "uteroglobin-like" protein includes those isolated from mouse, rat, rabbit, etc., having substantially the same amino acid sequences and/or substantial sequence similarity, termed conservative substitutions, with native human uteroglobin. With regard to sequence similarity, like-amino acids may be substituted in a uteroglobin-like protein, e.g. tyrosine for phenylalanine or glycine for alanine. Uteroglobin-like proteins which are considered substantially similar have approximately 30% sequence similarity, preferably 50% sequence similarity, more preferably at least 75% sequence similarity, and most preferably at least 90-95% sequence similarity. Uteroglobin-receptor ligands are peptide, protein or chemical moieties (e.g. organic ligands) that bind to the uteroglobin receptor and mediate all or part of its activities. Uteroglobin structural analogs are compounds, peptides or proteins, or fragments or derivatives thereof having substantially similar secondary and tertiary structural characteristics when compared to native uteroglobin, such that a structural analog retains at least 50% and preferably at least 75% of the activity of native protein. In a most preferred embodiment, a structural analog retains at least 90% of the activity of the native protein and retains the ability to interact with the uteroglobin receptor and to mediate all or part of its activities.

Further, the uteroglobin used in the method of the present invention is substantially pure. The term "substantially pure" refers to uteroglobin having a purity of about 75% to about 100%. In a preferred embodiment, uteroglobin has a purity of about 90% to about 100%, and in the most preferred embodiment, uteroglobin has a purity of at least 95%.

In so much as the present invention provides a method of treating or preventing a disease condition associated with fibronectin deposition, lung damage, and/or decreased lymphocyte production, the term "prevention" refers to preventing the development of disease in a susceptible or potentially susceptible population, or limiting its severity or progression, whereas the term "treatment" refers to the amelioration of a disease or pathological condition.

The Interaction Between Uteroglobin and Lung Damage, Lymphocyte Production, and Fibronectin

Using neonatal lambs delivered by caesarean section as a model of surfactant-dependent neonatal respiratory distress syndrome (RDS), it was found that recombinant human uteroglobin did not interfere with surfactant replacement therapy. In fact, an animal with severe meconium aspiration which potently inactivates surfactant responded to the administration of recombinant human uteroglobin by a marked increase in blood oxygenation and pH, both of which are indicators of improved lung function. This was the first observation of a direct effect of uteroglobin on lung tissue.

It was also unexpectedly found that the bioavailability of recombinant human uteroglobin in this experiment was excellent. These results show that recombinant human uteroglobin may be administered systemically via the lungs, for the purpose of raising circulating levels of the protein, to deliver the protein to tissues and organs, and to raise the concentration of uteroglobin in the urine. Therefore, it is possible to treat various internal organs and tissues, including the vasculature, muscle, connective tissue, bone, blood cells, stomach, kidneys, pancreas, liver, intestines, colon, heart, spleen, thymus, uterus, and bladder, by administering uteroglobin topically to the lungs through intratracheal deposition or through an inhaler or nebulizer. Further, results obtained from the intravenous, intranasal, and stomach gavage administration of uteroglobin to adult Wistar rats indicated that these routes may be practical for the systemic administration of the protein in humans. The presence of radioactive recombinant human uteroglobin in protein extracts of the trachea, bronchi, esophagus, and thyroid in an animal administered uteroglobin via each of these routes show that these tissues take up uteroglobin from the circulatory system. This demonstrates that one route of administration may be effective in the specific delivery of protein to target delivery for another system, e.g., the digestive system.

Next, using a ventilated newborn piglet, a well-characterized model of neonatal lung injury, one can readily observe significant decreases in pulmonary compliance and lung function, as well as increases in inflammatory markers which are indicative of pulmonary inflammation. These animals were administered uteroglobin, then broncho-alveolar lavage fluid (BAL), serum, and urine were collected in order to monitor the half-life and elimination of uteroglobin following intratracheal administration. Further, the total protein concentration in BAL samples was measured

and total soluble protein was calculated. These parameters are significant indicators of lung injury. The data show that administration of recombinant human uteroglobin increased pulmonary compliance in the newborn piglets sampled, and that the effect was independent of surfactant function. Complete blood counts (CBC) and differential cell counts were taken on the blood samples collected prior to and following uteroglobin administration to the ventilated newborn piglets. It was found that a single dose of recombinant human uteroglobin in the newborn piglets significantly enhanced lymphocyte proliferation and decreased polymorphonuclear leukocytes production.

Therefore, the present invention provides methods and compositions for improving and/or normalizing lung function, pulmonary compliance, blood oxygenation and/or blood pH. Suitable compositions include uteroglobin, and preferably recombinant human uteroglobin, in a dosage of 10 ng/kg - 25 mg/kg. The methods may be used to treat patients suffering from reduced lung function and/or pulmonary compliance as a result of exposure to non-atmospheric gases, non-atmospheric ratios of atmospheric gases, inhaled chemicals, pollutants, irritants, inhaled pollens, allergens, particulate matter, and airborne infectious agents.

The present invention also provides methods and compositions for increasing lymphocyte production *in vivo*, increasing the production of suppressor T cells, enhancing a lymphocyte-mediated response *in vivo*, and decreasing the production of polymorphonuclear leukocytes. The method of the present invention contemplates a dosage of uteroglobin, preferably recombinant human uteroglobin, of 1 ng/kg - 100 mg/kg. The lymphocytes that are typically affected by this method are effector lymphocytes and cytotoxic T cells, and more particularly, helper T cells, plasma B cells, and memory B cells. Further, in so much as the present invention provides a method of enhancing a lymphocyte-mediated response *in vivo*, such a method may be used to enhance the effects of the administration of a vaccine, such as a B cell or T cell vaccine, or a tolerance-inducing treatment, such as oral tolerance or allergy shots.

Finally, the interaction between uteroglobin and fibronectin was examined. It was found that recombinant human uteroglobin was a potent inhibitor of cellular adhesion to fibronectin and that it specifically bound to type III domains of fibronectin. Such domains are present in nearly all protein components of the extracellular matrix, e.g., laminin, collagens, vitronectin, and fibrin, as well as in numerous membrane bound proteins, including adhesion molecules, integrins, and receptors. Therefore, the inhibition of cellular adhesion by uteroglobin indicates that uteroglobin

can play a critical role in cell-cell and cell-extracellular matrix interactions.

Therefore, the present invention provides methods and compositions for inhibiting the following processes: (1) fibronectin-dependent cell adhesion to fibronectin, (2) interactions between fibronectin and cells dependent on fibronectin binding, (3) inflammatory cell and fibroblast migration on fibronectin already deposited *in vivo*, (4) fibronectin-dependent cell adhesion *in vivo*, and (5) an interaction between a cell having a PLA_2 receptor and an extracellular matrix protein and/or membrane bound protein comprising at least one fibronectin type III domain. The compositions contain uteroglobin, and preferably recombinant human uteroglobin, in a dosage of 8 μg-3.5 g total dose per 70 kg patient, and more preferably, 25 ng/200 μl - 10 μg/200 μl.

Cells dependent on fibronectin binding include, but are not limited to, neural cells, muscle cells, hematopoietic cells, fibroblasts, neutrophils, eosinophils, basophils, macrophages, monocytes, lymphocytes, platelets, red blood cells, endothelial cells, stromal cells, dendritic cells, mast cells, and epithelial cells. A patient suffering from one of the following conditions may benefit from therapies which inhibit the formation of vascular adhesions following surgery, atheroschlerosis, thrombosis, heart disease, vasculitis, formation of scar tissue, restenosis, phlobitis, COPD (chronic obstructive pulmonary disease), pulmonary hypertension, pulmonary fibrosis, pulmonary inflammation, bowel adhesions, bladder fibrosis and cystitis, fibrosis of the nasal passages, sinusitis, inflammation mediated by neutrophils, and fibrosis mediated by fibroblasts.

In so much as the invention provides a method of inhibiting inflammatory cell and fibroblast migration on fibronectin already deposited *in vivo*, inflammatory cells targeted by this method include, but are not limited to, neutrophils, eosinophils, basophils, macrophages, monocytes, lymphocytes, platelets, red blood cells, dendritic cells, mast cells, and fibroblasts.

Finally, extracellular matrix proteins affected by the method of the present invention include, but are not limited to, laminin, collagen, vitronectin, and fibrin, and membrane bound proteins affected by the instant method include, but are not limited to, adhesion molecules, integrins, and receptors.

Without wishing to be bound by any particular theory, the interactions between uteroglobin and lung damage, lymphocyte production, and fibronectin do not appear to be discrete and insular occurrences. Rather, they are indicative of a complex network of interactions between a variety of physiological events. First, the primary site of action of surfactant phospholipids in the lungs is thought to be the alveoli, which are membranous sacs at the end of small passages called

bronchioles. The surfactant allows the alveoli to expand and fill with air in response to the expansion of the chest wall which is mediated by smooth muscle. Surfactant also mediates O_2-CO_2 gas exchange across the mucosal fluid layer and alveolar membranes. The smooth muscle components of the lungs, i.e., the endothelial cell layers of bronchi, bronchioles, blood vessels, and capillaries are not known to be affected by surfactant.

The effect of uteroglobin on pulmonary compliance and serum protein leakage into BAL fluids in piglets indicate that a second mechanism of action for uteroglobin is at work, and one that is distinct from the inhibition of PLA_2-mediated digestion of surfactant phospholipids. First, the observation that piglets ventilated with 100% oxygen that received uteroglobin had normalized lung compliance in comparison to those similarly ventilated but not administered uteroglobin indicates that uteroglobin mediates an entirely unanticipated effect on the lungs that is distinct from its protective effects on exogenous surfactant. Further, there was no significant difference in surfactant function in BAL of animals that did and did not receive uteroglobin. This shows that the uteroglobin-mediated protection of surfactant phospholipids from digestion by soluble, secreted PLA_2s did not persist 48 hours after administration. In contrast, the uteroglobin-mediated effect on pulmonary compliance did persist 48 hours after administration. Thus, at the 48-hour endpoint, surfactant function did not correlate with the uteroglobin-mediated differences in pulmonary compliance. Therefore, uteroglobin mediates a surfactant-independent effect on pulmonary compliance. In accordance with these observations, uteroglobin could be described as a bronchodialator.

Because surfactant improves pulmonary compliance primarily by making the alveolar sacks more elastic, it follows that uteroglobin affects the flexibility of the other primary structures involved in pulmonary compliance, i.e., the bronchi and bronchioles. Bronchi and bronchioles are composed of three main cellular layers: the surface epithelia, the stroma, and the endothelia. The endothelial layers contain the smooth muscle responsible for the changes in volumetric capacity of the bronchi and bronchioles. Therefore, uteroglobin most likely affects pulmonary compliance by increasing the ability of smooth muscle to expand and contract. This explanation is supported by the observation that the protein content of the BAL of uteroglobin-treated piglets is significantly lower than that of untreated piglets. The source of excess protein in BAL fluids is generally the serum. The amount of protein that leaks from the serum into the BAL depends upon local vascular permeability. Pro-inflammatory treatments to the lungs, such as 100% oxygen exposure, generally

increase vascular permeability, resulting in excess protein in BAL fluids. The administration of uteroglobin countered this effect. Vascular permeability is, in part, dependent upon the degree of smooth muscle contraction in blood vessels and smooth muscle contraction is controlled by the autonomic nerve system.

Fibronectin type III repeats are found in structural proteins of the musculature, such as collagen, titins, and tenascins, as well as in several cellular adhesion molecules, such as ICAM-1 (intercellular cell adhesion molecule), LFA (leukocyte function associated antigens), VCAM-1 (vascular cell adhesion molecule), and NCAMs (neural cell adhesion molecules).

There are 17 type III repeats in fibronectin that can form intramolecular and intermolecular bridges and their formation may be calcium dependent. Thus, the type III repeats can act like building blocks that fit together. When fibronectin mediates fibrillogenesis, these repeats may interact via hydrophobic and ionic interactions, acting like beads on a strand that can interlock and align. The strings may be locked into threads by the action of transglutaminase which can covalently crosslink the fibronectin strands together and build the fibril. Therefore, one can assume that fibronectin type III repeats in molecules other than fibronectin may also interact non-covalently to affect communication. For example, the interaction between fibronectin type III repeats in neural cell adhesion molecules and fibronectin type III repeats in muscle structural proteins like collagen, titins, and tenascins, could mediate translation of the electrochemical signal traveling down the neuronal axon into the trigger for muscle contraction. Alignment of the fibronectin type III repeats, therefore, provides a physical connection between nerve and muscle, and interactions between fibronectin type III repeats may be important to the normal function of the lungs. The enhanced pulmonary compliance in uteroglobin-treated piglets shows that uteroglobin binds fibronectin type III repeats block the alignment of type III repeats in both nerve and muscle *in vivo*, perhaps regulating the degree of muscle contraction by sterically blocking the alignment of excess fibronectin type III repeats, in muscle tissue and at the interface of muscle tissue and neural tissue.

Preferred Routes of Administration and Formulations for Uteroglobin

Uteroglobin may be administered either alone or in combination with other active agents or compositions typically used in the treatment or prevention of the above-identified disease conditions. Such active agents or compositions include, but are not limited to steroids, non-steroidal anti-inflammatories drugs (NSAIDs), chemotherapeutics, analgesics, immunotherapeutics,

antiviral agents, antifungal agents, vaccines, immunosuppressants, hematopoietic growth factors, hormones, cytokines, antibodies, antithrombotics, cardiovascular drugs, or fertility drugs. Also included are vaccines, oral tolerance drugs, vitamins and minerals.

Uteroglobin may be administered to target a uteroglobin-receptor. Targeting of a uteroglobin receptor refers to inducing specific binding of a ligand to a receptor to mediate effects on cell growth.

As discussed above, the data show that recombinant human uteroglobin may be administered systemically via the lungs, for the purpose of raising circulating levels of the protein, to deliver the protein to tissues and organs, and to raise the concentration of uteroglobin in the urine. Therefore, one can treat various internal organs and tissues, including the vasculature, muscle, connective tissue, bone, blood cells, stomach, kidneys, pancreas, liver, intestines, colon, heart, spleen, thymus, uterus, and bladder, by administering uteroglobin topically to the lungs through intratracheal deposition or through an inhaler or nebulizer. Further, the data also show that intravenous, intranasal, and stomach gavage administration of uteroglobin are practical for the systemic administration of the protein in humans.

Uteroglobin may be administered intravenously or, in the case of treatment of neonatal RDS/BPD and adult RDS, in the form of a liquid or semi-aerosol via the intratracheal tube. Other viable routes of administration include topical, ocular, dermal, transdermal, anal, systemic, intramuscular, slow release, oral, vaginal, intraduodenal, intraperitoneal, and intracolonic. Such compositions can be administered to a subject or patient in need of such administration in dosages and by techniques well known to those skilled in the medical, nutritional or veterinary arts taking into consideration such factors as the age, sex, weight, and condition of the particular subject or patient, and the route of administration. The compositions of the present invention may also be administered in a controlled-release formulation. The compositions can be co-administered or sequentially administered with other active agents, again, taking into consideration such factors as the age, sex, weight, and condition of the particular subject or patient, and, the route of administration.

Further, the data show that recombinant human uteroglobin may be administered systemically via the digestive tract (orally), for the purpose of raising circulating levels of the protein, to deliver the protein to tissues and organs, and to raise the concentration of uteroglobin in the urine. Therefore, one can treate various internal organs and tissues, including the lungs,

vasculature, muscle, connective tissue, bone, blood cells, stomach, kidneys, pancreas, liver, intestines, colon, heart, spleen, thymus, uterus, prostate, and bladder by administering uteroglobin topically to the lungs through a drink, pill, nutraceutical, or suppository.

Examples of compositions of the invention include edible compositions for oral administration such as solid or liquid formulations, for instance, capsules, tablets, pills, and the like liquid preparations for orifice, e.g., oral, nasal, anal, vaginal etc., formulation such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable administration), such as sterile suspensions or emulsions. However, the active ingredient in the compositions may complex with proteins such that when administered into the bloodstream, clotting may occur due to precipitation of blood proteins; and, the skilled artisan should take this into account.

In such compositions uteroglobin may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, DMSO, ethanol, or the like. uteroglobin can be provided in lyophilized form for reconstituting, for instance, in isotonic aqueous, saline, glucose, or DMSO buffer. In certain saline solutions, some precipitation of recombinant human uteroglobin has been observed; and this observation may be employed as a means to isolate inventive compounds, e.g., by a "salting out" procedure.

Further, the invention also comprehends a kit wherein uteroglobin is provided. The kit can include a separate container containing a suitable carrier, diluent or excipient. The kit can include an additional agent which reduces or alleviates the ill effects of the above-identified conditions for co- or sequential-administration. The additional agent(s) can be provided in separate container(s) or in admixture with uteroglobin. Additionally, the kit can include instructions for mixing or combining ingredients and/or administration.

EXAMPLES

The invention will now be further described with reference to the following non-limiting examples. Parts and percentages are by weight unless otherwise stated.

Example 1

Recombinant human uteroglobin was administered to several mammalian species via several routes of administration to determine the safety and biological activity of the protein. The protein was given to rats in order to assess pharmacokinetics, bioavailability, and tissue distribution when administered intravenously, intranasally, and by stomach gavage. It was also given intratracheally to very young animals of three large animal species, including premature baboons, premature lambs and newborn piglets. The biological activity of recombinant human uteroglobin and its effect on various aspects of lung function was evaluated in these animal studies. The concentrations of recombinant human uteroglobin in all species were determined using an ELISA assay that is specific for human uteroglobin.

a. Purification of Recombinant Human Uteroglobin

Recombinant human uteroglobin was cloned and expressed by a method similar to that described in copending U.S. application serial no. 08/864,357.

In the alternative, the protein was extracted from the *E. coli* cell paste by high pressure shear and clarified by centrifugation. The first crude fraction was separated by tangential flow filtration at 100,000 daltons, followed by chromatography on anion exchange and hydroxyapatite supports. The purified protein was further purified by tangential flow filtration at 30,000 daltons.

b. ELISA for uteroglobin

A quantitative competitive ELISA was developed that provides the necessary sensitivity and precision required for measurement of recombinant human uteroglobin in human samples for use in clinical trials. The assay utilizes a rabbit polyclonal anti-human urine protein-1 (uteroglobin) antibody obtained from Dako, USA. Coating and blocking conditions, antibody dilution, and the type of microtiter plate have been optimized for signal reproducibility and for stability in storage.

Antibody (supplied as a 2mg/ml stock solution) was used to coat the wells of microtiter plates at a 1:2,500 dilution in a 0.1M carbonate/bicarbonate buffer, pH 9.5. Pipetting of all reagents into microtiter wells was done with an 8-or 12- multichannel pipetter. The diluted antibody solution was pitpetted into the wells of Nunc Maxisorp strip plates (100 microliters/well), then the plate strips was sealed in Ziploc bags, and incubated overnight at room temperature (18-25°C).

The next day, the coating solution was removed from each well by aspiration and 200 microliters of blocking buffer (5% sucrose, 5% Bovine serum albumin in phosphate buffered saline) was added to each well. Plate strips were placed back into Ziploc bag and onto a benchtop rotator and rotated gently for 2 hours at room temperature. After the two hour blocking step, the contents of all wells were aspirated and the plate strips were placed upside down in a biosafety cabinet with the fan on, to dry for two hours. Dry plates were stored at 4°C until ready to use for the assay. Plates prepared in this manner are stable for at least ten weeks.

In this competitive immunoassay format, the anti-uteroglobin antibody is used as the capture reagent for any uteroglobin in the sample. A conjugate of horse radish peroxidase (HRP) to recombinant human uteroglobin was generated using a Pierce HRP-labelling kit. The uteroglobin-HRP conjugate bound to the anti-uteroglobin antibody coating the wells, and generated a signal (A_{490}) proportional to the amount of HRP-uteroglobin conjugate bound. Signal was generated using a standard HRP enzymatic colorimetric reaction (Pierce OPD substrate). An optimized amount of the uteroglobin-HRP conjugate was mixed with a sample to be assayed (which may be pre-diluted with PBS, if necessary). Typically, two different dilutions (1:2-3 and 1:10) of each sample were run in duplicate, requiring ~ 100 microliters of sample. The assay thus revealed a decrease in signal as the uteroglobin in the sample competes with the uteroglobin-HRP conjugate for antibody binding sites, as shown in Figure 1. A standard curve, using carefully quantitated recombinant human uteroglobin calibrators, was always run in duplicate with each set of samples to assess reproducibility and quantitate uteroglobin in samples.

The uteroglobin ELISA involves a single antibody binding step to capture uteroglobin antigens in each sample. Therefore, the sample must be pre-mixed with the uteroglobin-HRP in an untreated microtiter dish, prior to addition to antibody-coated wells. A 110 microliter volume of recombinant human uteroglobin calibrator, pre-diluted sample or control (PBS only) was mixed with 110 microliter of the uteroglobin-HRP conjugate (1:200 dilution in PBS of 1 mg/ml stock solution) in appropriate wells of a pre-labeled microtiter dish). Once the samples were prepared, the entire 220 microliters was transferred to labelled antibody-coated wells. The microtiter plate strips were incubated at room temperature for 60-75 minutes on a benchtop rotator to allow uteroglobin antigen to bind to the wells. Plates were then washed three times using a microplate washer (Biotek Instruments model) with 0.05% Tween-20 in PBS. The HRP substrate (Pierce OPD) was prepared according to the manufacturer's instructions. Substrate (100 microliters) was

then added to all test wells using a multi-channel pipetter and the plate was incubated for 30 minutes at room temperature. Color development was stopped at 30 minutes by adding 50 microliters of 1.2N sulfuric acid to each well. Absorbance was read in a microtiter plate reader (Biotek Instruments EL800) at a wavelength of 490 nm.

A uteroglobin standard curve, uteroglobin sample concentration and test statistics were generated with KC4 software (Biotek Instruments) through a direct interface were between the microplate reader and PC computer. Coefficients of variation (CV) in this assay between less than 8% at concentrations down to 25 ng/ml and were generally lower than 15% between concentrations of 1-25 ng/ml with recombinant human uteroglobin calibrators and in human sera.

c. Intratracheal Administration of Recombinant Human Uteroglobin to Pre-Term Lambs

This study used neonatal lambs delivered by cesarean section as a model of surfactant-dependent neonatal respiratory distress syndrome (RDS). The primary objective of the study was to determine whether the administration of recombinant human uteroglobin would in any way decrease the benefit of surfactant administration in these animals. Since uteroglobin is known to bind to phospholipids, it could thereby interfere with the action of surfactant, which is mainly composed of phospholipids. The severity of RDS in the animals required very aggressive ventilation by human clinical standards, and thus provided a sensitive indication of any effects of recombinant human uteroglobin on the action of surfactant in RDS rescue.

Four lambs were delivered between 135-138 days of gestation by cesarean section and were in respiratory distress at birth. One dose of surfactant was administered after each animal was stabilized on ventilation, and 15 minutes later approximately 5mg/kg recombinant human uteroglobin (in two animals) or control vehicle (in the remaining two animals) was administered.

Although all animals were of similar gestational age, body weight ranged from 2.6 to 3.6 kg, and condition at birth ranged from mild to severe respiratory distress. The three lowest weight animals (#1, #2 and #4) had severe or very severe RDS, while animal #3 had milder RDS as judged from the initial chest x-ray. This was also reflected in the pre-treatment blood oxygenation (paO_2), which was considerably better in animal #3 than in the other three animals. Animal #4 was exceptional in that it was born with very severe meconium aspiration. The amniotic fluid was a thick brown consistency and tracheal aspirates from this animal were also brown and very viscous.

There were no apparent physical defects but the animal was not active prior to sedation. Chest X-ray showed that the animal had the most severe respiratory distress, with mostly opaque lungs. The two animals treated with recombinant human uteroglobin had the most severe RDS, despite the fact that one of them had severe meconium aspiration with mostly opaque lungs.

All animals received the same surfactant treatment, except that animal #1 received only one dose of surfactant (shortly after birth), whereas the others received two (the first shortly after birth and the second six hours later). Animal #1 required very high ventilator pressure (PIP $\geq$ 50) to maintain blood oxygenation and developed pneumothorax (confirmed by chest X-ray) at 10.5 hours after delivery. When final fluid samples were taken the animal was euthanized. After treatment of this animal, the protocol was altered to provide for a second dose of surfactant and recombinant human uteroglobin six hours after the first doses in accordance with prescribing information for Survanta. The treated animals were #2 (5 mg/kg x 2) and #4 (6.25 mg/kg x 2). Animals #1 and #3 did not receive the study drug; animal #1 received no treatment and animal #3 received a volume of normal saline equal to the volume of recombinant human uteroglobin solution given to the treated animals.

All four animals responded as anticipated to surfactant with increases in paO_2/FiO_2, decreases in $paCO_2$ and increases in blood pH and bicarbonate excess, BE(B). These data are shown in the Figures 2-5 representing blood values as a function of time in each animal. The animal (#2) treated with recombinant human uteroglobin (but without meconium aspiration) showed the greatest response to surfactant administration based on blood gases, even though it had more severe respiratory distress than the animals not treated with recombinant human uteroglobin. The other animal treated with recombinant human uteroglobin, which had meconium aspiration (#4), had significantly delayed responses in paO_2 relative to the other animals (about 180 minutes versus 15-30 minutes), but eventually responded better than the untreated animals. The two treated lambs also developed a higher blood pH relative to the untreated animals, and were the only animals in the study to reach a positive bicarbonate excess. None of the animals received electrolytes or any fluids that might have resulted in induction of a metabolic alkalosis, so the increases in blood pH and bicarbonate excess were due solely to improvements in pulmonary function mediated by recombinant human uteroglobin.

Table 1. Summary of Animals Treated				
Single/twin	Animal #1 Single	Animal #2 Twin (with #3)	Animal #3 Twin (with #2)	Animal #4 Twin (other dead)
Sex	Male	female	Female	Female
Gest. Age (days)	134	136	136	135
Weight (kg)	3.1	2.7	3.6	2.6
Condition at birth	Vigorous Severe RDS	Active Severe RDS	Active Mild RDS	Not active Severe RDS
Amniotic fluid	Clear	Clear	Clear	Meconium
Pre-treatment Pa_{02} (Torr)	50	80	160	90
Pre-treatment chest x-ray	Significant opacity Poor inflation	Significant opacity Poor inflation	Mostly clear Good Inflation	Mostly opaque Poor Inflation
Survanta @ birth	200 mg (65 mg/kg)	200 mg (74 mg/kg)	200 mg (55 mg/kg)	200 mg (77mg/kg)
@6 hrs	Not treated	200 mg (74 mg/kg)	200 mg (55 mg/kg)	200 mg (77 mg/kg)
Recombinant human uteroglobin *@ birth	Not treated	6.25 mg/kg	0 mg/kg (saline)	5.0 mg/kg
@ 6 hrs	Not treated	6.25 mg/kg	0 mg/kg (saline)	5.0 mg/kg
Post-treatment	200 @ 120 min.	280 @ 100 min.	380 @ 100 min.	450 @ 300 min.
Pa_{02} (Torr Survival)	11 hrs	12 hrs	12 hrs	12 hrs
Cause of death	Pneumothorax	Euthanized	Euthanized	Ethuanized

It is clear from this data that recombinant human uteroglobin did not interfere with surfactant replacement therapy and is thus safe to use during surfactant rescue therapy. The animal with severe meconium aspiration responded remarkably well with respect to blood oxygenation and pH, indicators of improved lung function, in the presence of recombinant human uteroglobin. Meconium is known to inactivate surfactant and this animal did not respond to the exogenous surfactant in the normal timeframe of 15-30 minutes, as did the other animals. Therefore, the delayed blood gas responses that each occurred about 180 minutes after each administration of surfactant and recombinant human uteroglobin can be attributed to an independent action of recombinant human uteroglobin on the lung tissue. This is the first observation of the direct effect of uteroglobin on lung tissue.

Lambs were treated and monitored for twelve hours post-delivery. Bodily fluid samples were taken from each animal for analysis of uteroglobin concentration by ELISA as listed in Table 2, below.

Table 2: Samples for Uteroglobin Pharmacokinetics

	Serum	Plasma	Tracheal Aspirate	Urine
Pre-Treatment	X	X		X
2 Hours			X	
6 Hours	X	X		X
12 Hours			X	X

After sacrifice at twelve hours, necropsy tissue specimens, focusing on lungs, were examined for gross pathology and preserved in formalin for histopathological analysis. Animals treated with recombinant human uteroglobin showed no evidence of drug-related toxicity during treatment. Tissues and organs isolated from the animals showed no gross or microscopic abnormalities to recombinant uteroglobin administration. Therefore, intratrachael administration of recombinant human uteroglobin in at least two separate doses of up to 6.25 mg/kg was safe and non-toxic.

The concentration of recombinant human uteroglobin in the lamb fluids was quantiated by uteroglobin ELISA and results are shown in the following table. The recombinant protein followed the same pattern of distribution in bodily fluids s the native protein in humans. That is, it was taken up from the site of administration to the extracellular lung fluids into the blood, and was excreted in the urine. Therefore, the recombinant human protein behaves the same way *in vivo* as does the native protein.

Table 3. Recombinant Human Uteroglobin Concentration in Bodily Fluids (ng/mL)

Sample	Animal #1	Animal #2	Animal #3	Animal #4
Tracheal Aspirates				
Pretreatment	<5	<5	<5	47
1-2 Hrs Post-treatment	nd	>68,200	10	19,400
2-4 Hrs Post-treatment	<5	>68,200	11	nd
4-6 Hrs Post-treatment	nd	nd	nd	11,900
6-8 Hrs Post-treatment	nd	>68,200	nd	nd
8-10 Hrs Post-treatment	nd	13,000	nd	58,200
Plasma				
Pretreatment	nd	<5	<5	<5
6 Hrs Post-treatment	<5	4,090	<5	584
8 Hrs Post-treatment	nd	nd	nd	2,932
12 Hrs Post-treatment	<5	3,795	<5	1,964
Serum				

Pretreatment	<5	<5	<5	nd
2 Hrs Post-treatment	nd	nd	nd	723
6 Hrs Post-treatment	<5	3,455	<5	447
8 Hrs Post-treatment	nd	nd	nd	1,730
12 Hrs Post-treatment	<5	3,758	<5	1,563
Mother of animal	nd	nd	5.3	<5
Urine				
Pretreatment	nd	5.3	<5	8
6 Hrs Post-treatment	nd	31	<5	nd
Post-mortem (12 Hrs)	<5	27	<5	121
nd=not done				

The bioavailability of intratracheal recombinant human uteroglobin was excellent. The recombinant human uteroglobin-treated animals showed 12-60 μg/mL recombinant human uteroglobin in tracheal aspirates at two hours after the first recombinant human uteroglobin administration, 0.5-4.0 μg/mL in serum and plasma that peaked at four hours after the first recombinant human uteroglobin administration, and 5-100 ng/mL in urine at ten hours after the first dose of recombinant human uteroglobin. This demonstrates that recombinant human uteroglobin can be administered systemically via the lungs, for the purpose of raising circulating levels of the protein, for the purpose of delivering recombinant human uteroglobin to tissues and organs, and for the purpose of raising the concentration of uteroglobin in the urine. Thus, it is possible to treat various internal organs and tissues, including the vasculature, muscle, connective tissue, bone, blood cells, stomach, kidneys, pancreas, liver, intestines, colon, heart, spleen, thymus, ureters and bladder, etc. by administering uteroglobin topically to the lungs by intratracheal deposition or through an inhaler device or nebulizer.

d. Intratracheal administration of Recombinant Human Uteroglobin to newborn piglets ventilated for 48 hours

The ventilated newborn piglet was selected for this study because it is a well-characterized model of neonatal lung injury. While the newborn piglet is not a surfactant dependent model, it is an excellent model for neonatal lung injury mediated by oxygen toxicity arising from the use of positive pressure ventilation and elevated oxygen delivery in RDS rescue. Significant decreases in pulmonary compliance, as well as increases in inflammatory markers, indicative of pulmonary inflammation, are observed within 48 hours in this model. Although the model is not surfactant

dependent, it is quite responsive to the administration of exogenous surfactant. Thus, the linkage between increased pulmonary inflammation and decreased pulmonary mechanical function that occurs in human neonates who develop chronic lung disease is preserved in this model. Further, direct injury to pulmonary surfactant is measured by analyzing the surface tension properties of surfactant collected by BAL from the lungs of treated and untreated animals. Therefore, this model is well suited to the evaluation of the effect of uteroglobin on lung function and safety of intratracheal recombinant human uteroglobin for the treatment of RDS and the prevention of chronic lung disease in ventilated human neonates.

In this study, piglets were treated with combinations of artificial surfactant and recombinant human uteroglobin or control vehicle and then ventilated with either room air or 100% oxygen for 48 hours. Exogenous surfactant consisted of Survanta (Ross Labs) given intratracheally, in a single dose, via the endotracheal tube and at the recommended human dosage of 100 mg/kg. Recombinant human uteroglobin, formulated in sterile saline (0.9%), was given in a single intratracheal dose, within 30 minutes following the surfactant and at concentrations of 1, 5, and 25 mg/kg. Control groups received comparable volumes of sterile saline only.

A total of 40 piglets in ten groups of four will be analyzed in the completed study as shown in Table 4. The data provided herein represents the findings from 16 piglets. Half of the animals were ventilated with room air and half ventilated with 100% oxygen. Human neonates in respiratory distress always receive supplemental oxygen but it is often less than 100%, depending upon the degree of severity of the RDS and the medical practitioners' individual approach to ventilation management. Therefore, the use of room air and 100% oxygen allows the comparison of treatment extremes in this safety evaluation.

Table 4. Groups in the 48 Hour Study		
Recombinant human uteroglobin dose group	Ventilated with Room Air	Ventilated with 100% Oxygen
Control	N = 4	N = 4
Control vehicle + Survanta	N = 4	N = 4
1 mg/kg recombinant human uteroglobin + Survanta	N = 4	N = 4
5 mg/kg recombinant human uteroglobin + Survanta	N = 4	N = 4
25 mg/kg recombinant human uteroglobin + Survanta	N = 4	N = 4

Status of the 48 hour study: Most of the controls receiving Survanta and no recombinant human uteroglobin (7/8), as well as most of the animals receiving 25 mg/kg recombinant human uteroglobin plus Survanta (7/8) have been completed. (In fact an extra 25 mg/kg animal was added to the 100% oxygen group to establish safety.) No toxicity was observed in any of the animals receiving high dose recombinant human uteroglobin and a substantial benefit to pulmonary compliance was evident. The study will be completed at the low dose recombinant human uteroglobin groups.

i. Pharmacokinetics of Recombinant Human Uteroglobin in newborn piglets

In humans, a considerable body of evidence indicates that endogenous uteroglobin is produced in the pulmonary and tracheal epithelia, enters the blood by an unknown mechanism, and is eliminated from the blood via the kidney. Three types of fluid samples were collected in order to monitor the half-life and elimination of recombinant human uteroglobin following intratracheal administration. The first type was broncho-alveolar lavage fluid (BAL) collected at 48 hours, after sacrifice, lung excision and pulmonary function testing. This is as close to the site of administration as was practical to sample. The second type was serum collected before treatment and at 2, 4, 8, 12, 24, 36, and 48 hours after administration, from which an estimate of circulating half-life can be made. The third type of sample was urine, collected on the same schedule as serum. Overall, the distribution of recombinant human uteroglobin in piglet fluid was consistent with the distribution of endogenous uteroglobin in humans.

The concentration of recombinant human uteroglobin was measured in BAL, sera, and urine using the competitive ELISA described above. Only three sets of sera from seven untreated piglets were tested in order to establish the background level for the uteroglobin ELISA. The four remaining sets were not tested in the interests of conserving valuable ELISA reagents. Likewise, not all urine samples from untreated piglets were tested. Background immunoreactivity for the uteroglobin ELISA in serum ranged from undetectable to about 100 nanograms/ml and the highest background level in urine was 26 nanograms/ml.

The serum data were fairly consistent among the eight piglets dosed with recombinant human uteroglobin. Recombinant human uteroglobin was detected in all animals at the two hour timepoint. The recombinant human uteroglobin level peaked between two and eight hours post-administration. This shows that either the distribution of the drug in the lungs was variable or that the ability of the lungs to convey recombinant human uteroglobin to the blood was variable, or both. Peak serum levels of 4-17 μg/mL (mean 8.6±6.1 μg/mL) were measured within 2-8 hours of drug administration. Elimination of recombinant human uteroglobin from the serum corresponded well to first-order kinetics between 8 and 48 hours after drug administration (R^2=0.97) with a half-life of 7.9 hours. The half-life of uteroglobin in humans has not yet been accurately assessed. At 48 hours, serum uteroglobin levels were still elevated (0.24±0.16 μg/mL) relative to control animals, in which uteroglobin was usually less than the assay detection limit of approximately 0.01 μg/mL Data showing the concentration of recombinant human uteroglobin in serum as a function of time after intratracheal administration are shown in Figure 6.

A comparison of the urine recombinant human uteroglobin concentrations in the treated animals in the 100% oxygen group versus the room air groups shows that there may be a difference in the renal handling of circulating recombinant human uteroglobin. Animals #7, 31 and 42 seemed to excrete a much greater amount of recombinant human uteroglobin in their urine during the study period than did animals #12, 39, and 44. Piglet #22 appears to have been dehydrated since it did not produce much urine during the study period, was noted to have bloating and diarrhea within the first twelve hours of the study period, and the investigators noted difficulties extracting blood samples from the animal. It is not known whether this apparent difference in the renal handling of recombinant human uteroglobin reflects a difference in the molecular form of recombinant human uteroglobin or a difference in the kidneys' ability to process recombinant human uteroglobin. The kidney is known to respond to the level of oxygen in the blood and is part of the homeostatic

regulatory system, with potential feedback mechanisms to the lungs. An example of an altered molecular form of recombinant human uteroglobin that affects renal handling may involve complexing to a high molecular weight protein like fibronectin, which does not pass through the renal glomeruli. When homodimeric uteroglobin passes through the glomeruli, it is thought to be reabsorbed by the tubules, such that there is approximately a twenty to one hundred-fold steady state difference between the circulating concentration and the urine concentration in normal humans.

A considerable quantity of recombinant human uteroglobin also remained in the BAL fluid after 48 hours. The background level in the BAL of untreated piglets was about 200 nanograms/ml. This relatively high level of background was probably due to cross-reactivity with endogenous porcine uteroglobin or some other protein(s), as well as the matrix effects of this particular fluid in the uteroglobin ELISA. The BAL concentrations in treated piglets ranged from background levels of about 200 nanograms/ml (piglet #42) to over 16 micrograms/ml (piglet #31). The concentration of uteroglobin immunoreactivity in BAL from normal adult humans was 3-8 micrograms/ml with occasional concentrations up to 25 micrograms/ml in asymptomatic normal adults.

It is clear that a significant amount of recombinant human uteroglobin, remains in the extracellular lung fluids for an extended period of time (ie. two days). In particular, piglets #31 and #44 had significantly higher recombinant human uteroglobin in BAL than the rest of the treated piglets. The reason for this is not understood but there is no indication from either the lung pathology or the pulmonary function tests that these animals suffered any toxic effects as a result of high recombinant human uteroglobin immunoreactivity remaining in their lung fluids. These results also indicate that recombinant form of human uterglobin is most likely utilized and processed by the same pathways as the endogenous protein in the lungs, kidneys and circulatory system, based on parallels with published adult human data. Further, there is no indication that there is a significant difference between the pharmacokinetics of recombinant human uteroglobin in the newborn piglet versus that of endogenous uteroglobin in the adult human.

ii. Total Protein in BAL

Total protein concentration in BAL samples was measured and total soluble protein per right lung was calculated. The data are shown below in Table 5. A significant indicator of lung

injury is the concentration of total protein in lung lavage fluids. Serum proteins are known to leak into extracellular lung fluids when the alveolar-capillary barrier is impaired and/or vascular permeability is increased. There did not appear to be a significant difference in total protein among the four treatment groups. However, when data for all animals in the groups that received recombinant human uteroglobin are combined and compared to all of the animals in the groups that did not receive recombinant human uteroglobin, there was a significant difference (see table below).

Table 5.
Comparison of total protein in BAL

	No recombinant human uteroglobin * (n=6)	25 mg/kg recombinant human uteroglobin * (n=6)
Total protein (mean±S.D.)	426±144	318±200

The amount of total protein in the BAL fluids in six recombinant human uteroglobin treated animals was about 27% lower than in six untreated animals. This indicates that uteroglobin had a significant effect on regulation of vascular permeability, perhaps as a result of interactions with the smooth muscle component of the vasculature. This observation was consistent with the apparent benefit of recombinant human uteroglobin in pulmonary function tests, in that recombinant human uteroglobin-treated animals exhibited better lung compliance than their untreated counterparts.

Pulmonary function testing was successfully performed on nine of the fifteen animals studied, including at least two animals in each of the four groups. The data are shown in the accompanying figures. Figure 7 shows the pressure-volume relationships. While most of the animals (5) fall into a single main group, the animals with lower pulmonary compliance (piglet #11 and #43) are both in the 0 – 100 group. Of the two animals (piglet #23 and #31) that are above the pack with better lung compliance, both were ventilated with room air, one received recombinant human uteroglobin and one did not.

Figure 8 shows the mean pressure-volume relationships measured for animals ventilated with room air (error bars indicate standard deviation). There was no significant difference in lung compliance between room air-ventilated animals that did and did not receive the study drug (p=0.90).

Figure 9 shows the mean pressure-volume relationships measured for all animals ventilated with room air and 100% O_2 (error bars indicate standard deviation). There was clearly an improvement in mean pulmonary compliance in animals that were treated with recombinant human uteroglobin compared to saline controls.

In this model, most of the pulmonary damage manifested as decreased compliance is caused by hyperoxia rather than barotrauma. This effect can be seen in Figure 10, in which the mean pulmonary compliance among all four groups is compared. For animals not receiving the study drug, pulmonary compliance was considerably lower in animals ventilated with 100% oxygen (0100) relative to those ventilated with room air (0 RA) ($p=0.09$). For animals receiving the study drug, however, pulmonary compliance was similar whether or not the animals were ventilated with room air (25 RA) or 100% oxygen (25 100). Thus, recombinant human uteroglobin has countered the negative effects of 100% oxygen on pulmonary compliance.

e. Long term effects of intratracheal administration of Recombinant Human Uteroglobin to newborn piglets

Twenty newborn piglets were sedated, entubated and dosed according to the groupings shown in the table below. They were then allowed to recover and were maintained for one month. (Animals were bottle-fed for the first two weeks.) After 28 days the animals were sacrificed and necropsied for a full toxicological evaluation.

Several analyses were performed on piglets samples as shown in the following table. No evidence of toxicity was observed, even at the highest dose of recombinant human uteroglobin. There was no evidence of an anti-recombinant human uteroglobin antibody production at 28 days. Recombinant human uteroglobin did not persist in the circulation of the animals for 28 days, consistent with the 7.9 hour half-life measured in the 48 hour study. There was no long-term toxicity observed for intratracheal recombinant human uteroglobin administration in the newborn piglet.

Table 6.
28 Day Piglet Study Groups

Number of Piglets	Recombinant human uteroglobin dose	Survanta dose
4	0 mg/kg	0 mg/kg
4	0 mg/kg	100 mg/kg
4	1 mg/kg	100 mg/kg
4	5 mg/kg	100 mg/kg
4	25 mg/kg	100 mg/kg

Table 7
Summary of Samples and Analyses for 28 Day Piglet Study

Data or Sample Type	Type of Analysis	Pre-Rx	28 Days
Whole blood	CBC & Differentials	X	X
Serum	uteroglobin ELISA	X	X
Serum	Anti-uteroglobin antibody titer	X	X
Whole animal weights	Overall growth	X	X
Necropsy	Gross pathology		X
Organs in formalin: Lungs (both) Heart Liver Thyroid Adrenals (both) Spleen Kidneys (both) Brain Lymph nodes	Weights and histopathology		X

f. Piglet lymphocyte data

CBC (complete blood counts) and differential cell counts were done in the hospital lab on whole blood collected prior to uteroglobin administration and at 28 days post-administration, immediately prior to sacrifice. These data are shown in the table below. The mean and standard deviations of cell counts are given for the pre-treatment initial blook sample calculated for each piglet and the mean of the differences is shown for each group. There is a trend in the piglets in both groups that did not receive recombinant human uteroglobin towards lower PMN counts and higher lymphocytes counts at 28 days. This trend is normal in mammals since the arm of the immune system that promotes lymphocyte growth matures in the first year cells.

A single dose of recombinant human uteroglobin in the newborn piglets significantly enhanced both trends toward higher lymphocyte counts and lower PMN. The responses were somewhat dose dependent in that both increased dramatically between 1 and 5 mg/kg. But they decreased slightly at 25mg/kg which may indicate some type of saturation phenomenon at very high uteroglobin levels. However, it is clear that recombinant human uteroglobin significantly enhances lymphocyte proliferation, up to 2.5-fold, while it decreases PMN proliferation by up to 2.3-fold, over a one month period of time. The data further indicate that recombinant human uteroglobin may be a differentiation factor that shifts the development of stem cell precursors from the PMN lineage to the lymphocyte lineage.

Table 8: Blood Cell Counts (Mean±SD)					
Treatment Group Identifier	0 - 0 (n=4)	O - S (n=4)	1 - S (n=4)	5 - S (n=4)	25 - S (n=4)
White Blood Cells					
Initial	9.25±5.62	12.38±2.80	7.68±3.86	11.1±3.66	7.58±3.37
Final	6.35±1.53	8.20±3.18	7.08±2.24	5.83±0.53	7.83±1.11
(Final - Initial)=	-2.90	-4.18	-0.60	-5.27	0.25
Red Blood Cells					
Initial	4.39±0.58	4.30±1.00	4.87±0.55	4.24±0.79	4.42±1.13
Final	5.77±0.66	5.90±0.53	5.91±0.57	5.37±0.77	5.70±0.73
(Final - Initial) =	1.38	1.6	1.04	1.13	1.28
Hemoglobin					
Initial	9.13±0.69	8.93±1.92	10.13±0.99	8.45±1.38	8.98±1.92
Final	10.05±0.99	10.13±1.30	10.30±1.27	8.88±0.96	9.55±1.46
(Final - Initial) =	0.92	1.20	0.17	0.43	0.57
Hemacrit					
Initial	28.03±2.16	27.20±5.13	30.18±3.17	25.85±3.69	27.25±6.06
Final	30.38±3.10	30.73±3.62	30.58±3.21	26.23±3.50	27.78±5.48
(Final - Initial)=	2.35	3.53	0.40	2.62	0.53
Platelets					
Initial	459±161	518±45	389±46	458±55	502±94
Final	580±35	581±13	586±101	549±293	570±200
(Final - Initial) =	121	63	197	91	68
PMNs					
Initial	55.5±16.9	61.0±11.1	54.0±12.9	69.5±12.4	55.0±13.1
Final	39.8±16.9	46.7±0.6	42.8±7.5	33.8±16.0	31.5±16.0
(Final - Initial) =	-15.7	-14.3	-11.2	-35.7	-25.3
Lymphocytes					
Initial	38.3±16.9	30.5±11.2	42.5±11.9	26.0±10.4	36.0±13.4
Final	52.5±11.4	49.0±3.6	51.5±8.5	60.8±17.3	61.3±17.0
(Final - Initial) =	14.2	18.5	9	34.8	25.3
Monocytes					
Initial	3.50±2.38	4.50±2.65	2.75±2.50	2.50±1.73	5.33±6.66
Final	5.00±3.16	3.67±3.06	3.50±1.91	4.25±4.35	4.50±4.04
(Final - Initial) =	1.5	-0.83	0.75	1.75	-0.83
Eosinophils					
Initial	0.25±0.50	0.00±0.00	0.00±0.00	0.00±0.00	0.33±0.58
Final	0.50±1.00	0.00±0.00	0.75±0.96	0.00±0.00	0.00±0.00
(Final - Initial) =	0.25	0	0.75	0	-0.33
Bands					
Initial	0.25±0.50	0.25±0.50	0.25±0.50	0.50±1.00	0.00±0.00
Final	0.50±1.00	0.00±0.00	0.00±0.00	0.00±0.00	0.25±0.50
(Final - Initial)=	0.25	-0.25	-0.25	-0.50	0.25

g. Administration of recombinant human uteroglobin to Wistar rats

The primary purposes of the animal pharmacology and toxicology models used thusfar is to support the clinical trials of recombinant human uteroglobin in neonatal lung disease. However, they will also be supportive of studies of adult lung disease and provide certain basic information about the distribution, metabolism and excretion of the protein.

Uteroglobin is a naturally occurring mammalian protein for which there are no known post-secretory modifications, with the possible exception of alterations in oxidation state, depending upon whether zero, one or two of the possible disulfide bonds between the monomers of the dimeric protein are present. Therefore, it is very unlikely that biotransformation is a consideration in the pharmacodynamics of the recombinant human uteroglobin drug. The main source of endogenous uteroglobin protein in the body is the lungs and it is eliminated from the circulation by the kidneys. The tissue distribution or kinetics of elimination of recombinant human uteroglobin from the body has not when the recombinant protein drug is administered in doses representing a much higher level than the endogenous uteroglobin circulating in the body. But once the normal, circulating physiological concentration is reached (about 150 nanograms/ml in serum), then the elimination kinetics for recombinant protein drug can be expected to follow the pattern of the endogenous protein, provided the drug is biologically equivalent to the native protein.

The steady-state level of endogenous uteroglobin in the blood is 80-150 nanograms/ml and is 2-50 nanograms/ml in the urine of healthy individuals. Bernard, et al., showed that the kidney mediates the excretion of recombinant human uteroglobin from the blood into the urine by comparing serum and urine concentrations of native uteroglobin in normal healthy humans to those of patients with different types of renal impairment. The glomeruli of the kidney filter out small molecular weight waste products and proteins, with a size cutoff of approximately 40 kilodaltons. Uteroglobin is a very compact globular protein with a Stoke's radius of only 18.4 Angstroms, despite a molecular weight of 16 kilodaltons. Crystallization studies verify the very compact structure of this protein. In addition, uteroglobin will pass through a dialysis membrane with a molecular weight cutoff of 8.0 kilodaltons. Patients with either glomerular or tubular disease had high urine concentrations of uteroglobin, which was comparable to that of the serum in patients with the most severe renal disease, and human albumin injected intravenously in rats was reported to competitively inhibit tubular reabsorption of uteroglobin. Together, these results indicate that the uteroglobin homodimer may pass through the renal glomeruli, but is selectively reabsorbed by

the renal tubules prior to urinary excretion. Therefore, a significant proportion, as much as 30%, of the circulating endogenous uteroglobin may be removed by healthy kidneys and is excreted in the urine.

A pharmacokinetic study of recombinant human uteroglobin administration to rats was undertaken to provide basic information on half-life, tissue distribution, metabolism and excretion of the protein. A single dose of radiolabeled recombinant human uteroglobin was administered to a total of 12 Wistar rats by three routes of administration: intravenous, intranasal, and stomach gavage. Fluid samples were taken over a period of 24 hours, after which the animals were euthanized and dissected. Radioactivity was measured in all fluids and tissues.

Adult Wistar rats, ages 8-10 weeks, each received approximately 25 million dpm of highly-purified ^{125}I-labeled recombinant human uteroglobin (Lofstrand Labs, Inc.), corresponding to 1.9 μg of recombinant human uteroglobin or 13.6 μCi of ^{125}I (specific activity:7.18 μCi/μg protein). Four rats, two male and two female, were included in each of the three groups that received the ^{125}I-labeled recombinant human uteroglobin by the three routes of administration. Each animal received 7.17 – 8.64 μg/kg of recombinant human uteroglobin, corresponding to 51.3 – 61.6 μCi/kg of ^{125}I. Doses of recombinant human uteroglobin varied slightly between animals due to differences in body weight. Animals were housed in metabolism cages so that urine and feces could be collected for analysis at the end of the 24-hour study period. Blood (200-300 μL) was collected at 1,2,4,8, 12 and 24 hours after administration of the radiolabeled protein, and sera and plasma were prepared. The stomach gavage group were also sampled at 30 minutes after administration. Animals had free access to food and water throughout the study period. After final blood samples were collected, animals were exsanguinated and necropsied. In addition to the blood samples, 25 different tissue samples representing all major organs and tissues were collected. Samples were frozen at –80°C to preserve them for radiation measurements and protein extraction and analysis. Two animals died during the study, one apparently due to blood loss and the other apparently due to handling trauma. Neither death appeared related to the study drug.

Fluid and tissue samples were counted in a gamma counter to determine the amount of radioactivity present. Ten microliters of each sample of serum, plasma and urine were added to 3 mls of scintillation cocktail and counted for one minute each. The samples are analyzed using the competitive ELISA described above to determine concentrations of uteroglobin antigen in all fluid samples and protein extracts of tissues. Likewise, frozen organs were bisected and the intact half

was placed in a scintillation vial containing 3 mls of scintillation cocktail and counted for one minute each.

The remaining half of each organ sample was ground up in a 1.5 ml Eppendorf tube, using a motorized pestle (Fisher brand) and the powdered sample was resuspended in a protein extraction buffer (50 mM Tris-HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, 2 mM PMSF, 10 mM EDTA, 20 mg/ml each aprotinin, leupeptin, and pepstatin A) and vortexed briefly to mix. The mixture was then centrifuged in a microfuge at 4°C for 15 minutes. The supernatant was transferred to a clean tube and the pellet was discarded. SDS-PAGE analysis was performed on the supernatants, followed by autoradiogram to analyze the pattern of radioactive protein in the tissue samples.

Figures 11-13 show radioactive counts as a function of time for each of the three administration groups. The amount of radioactivity in the blood decreases over time, as expected, and appears to follow first-order elimination kinetics in the intravenous administration group. The elimination patterns in the intranasal and stomach gavage groups are not as distinct as that of the intravenous administration group, presumably due to absorption effects in the sinuses and gastrointestinal tract.

Radiation counts corresponding to recombinant human uteroglobin protein in these samples was confirmed by analyzing them with the uteroglobin ELISA that does not recognize free iodine or degradation products. These data are shown in Figures 14-16. In contrast to the radiation counts, there is a delay of 1-4 hours in reaching the peak uteroglobin concentration in the serum for the intranasal and oral routes. These data demonstrate that there is an uptake phase for uteroglobin across the mucosal surfaces of the stomach and upper respiratory tract. It is clear, however, that both the intranasal and oral routes of administration were effective in delivering a significant dose of the protein to the circulation. It is remarkable and unanticipated that these routes would be so effective for uteroglobin administration. The nasal mucosa is a barrier between the external flora containing bacteria and viruses, as well as inhaled antigens, and the internal system. Likewise, the gut (via the stomach) is also a selective barrier that distiguishes between food molecules and other ingested materials that are not food. These barriers are generally not permeable to molecules as large as uteroglobin and the specific and efficient uptake shows that there is an active or passive transport system that allows entry of uteroglobin in both the respiratory tract and the digestive tract.

Results in the intranasal group are also consistent with pharmacokinetics observed in both the neonatal lamb and piglet studies, in which uteroglobin persists for an extended period of time in

the extracellular lung fluids. This indicates that the extracellular mucosal fluid of the mammalian respiratory tract may serve as a reservoir from which the protein enters the blood. Inhalation may thus be a practical route for systemic administration of the protein in humans. The presence of radioactive recombinant human uteroglobin homodimer in protein extracts of trachea, bronchi, esophagus, and thyroid in an animal from each administration route show that these tissues take up uteroglobin from the circulatory system. This demonstrates that one route may be effective in the specific delivery of protein to target delivery for another system. For example, the intravenous route may be used to deliver a large dose of uteroglobin specifically to the gut via the esophagus or to the lungs via the trachea. Likewise, inhaled uteroglobin may be used to deliver uteroglobin to the gut and kidneys and oral uteroglobin may be used to deliver the protein to the lungs and kidneys. In summary, the data indicate that recombinant human uteroglobin enters the circulation by all three routes of administration tested proving the feasibility of intranasal and oral uteroglobin administration in humans.

Example 2

The demonstration of the binding interaction between human fibronectin and recombinant human uteroglobin prompted the development of a non-radioactive assay for this interaction that could be used as a measure of recombinant human uteroglobin biological activity. Therefore, two ELISA-based assay formats for the uteroglobin-fibronectin binding interaction were tested, as shown in Figures 17A-17B. Briefly, in the first of these assay methods recombinant human uteroglobin was used to coat the wells of a microtiter dish, which followed by fibronectin binding and detection of the bound fibronectin with an anti-fibronectin monoclonal antibody (Life Technologies, Inc.; product #12062-014). In the second assay, purified human fibronectin was used to coat the wells of a microtiter dish, followed by recombinant human uteroglobin binding and detection of the bound recombinant human uteroglobin with an anti-uteroglobin antibody (Dako, USA). Both formats gave comparable results with a 2-3 fold signal increase over controls when similar concentrations of recombinant human uteroglobin and hFn were used. There being no apparent advantage of one format over the other, the second format was chosen for further development and analyses due to the relative ease of reagent availability. A detailed description of the second ELISA assay follows.

Purified human fibronectin (hFn) was obtained as a frozen lyophil from Life Technologies, Inc. (Gaithersburg, MD), product #33016-015. According to the supplier's product specifications, this hFn preparation was purified from human plasma. The hFn was resuspended in sterile water to a concentration of 1 mg/ml. This stock solution was then aliquoted and unused aliquots were frozen at -80°C. An aliquot of the 1 mg/ml stock solution was diluted to 10 micrograms/ml with phosphate buffered saline (PBS) (pH 7.4). Fifty microliters of the diluted hFn were used to coat wells of a microtiter plate (Falcon) with 5 micrograms/well. Further dilutions were made to generate a set of wells containing 1 microgram and 500 nanograms of hFn as well. The hFn coating was performed overnight at room temperature. After coating, the hFn was removed by aspiration and the wells were blocked with a 1:1 dilution of Pierce blocker™ in PBS (pH 7.4), with a final concentration of 5% BSA in 1X PBS (pH 7.4). The blocking reagent was diluted immediately prior to the addition of 320 microliters per well. The plate was incubated for two hours at room temperature with gentle shaking. The blocking reagent was removed by aspiration and the wells were washed three times with 1X PBS (pH 7.4).

Purified recombinant human uteroglobin, diluted from a 10 mg/ml stock solution in PBS, was added to the wells in a constant volume of 50 microliters but in different concentrations, ranging from 1 ng/well to 1 microgram/well. After the addition of recombinant human uteroglobin or PBS controls, the plates were gently shaken for one hour at room temperature. The recombinant human uteroglobin was then aspirated off and the wells were washed three times with PBS and blocked again for an hour with undiluted Pierce blocker™ at room temperature. After re-blocking for an hour, the block buffer was removed by aspiration and the wells washed three times with 1X PBS, pH 7.4. Two hundred microliters of a 1:2,000 dilution of a rabbit polyclonal anti-human urine protein-1 (uteroglobin) antibody (Dako, USA) in PBS, were then added to each well. The plate was gently shaken for 1 hour at room temperature, then the antibody solution was removed by aspiration the plate was washed three times with PBS, and blocked again for one hour as described above.

A goat anti-rabbit IgG antibody, conjugated to horse radish peroxidase (HRP), was used to quantitate the amount of anti-uteroglobin antibody in the wells. Two hundred microliters of a 1:20,000 dilution of the HRP-conjugate in PBS was added to each well and the plate was shaken gently for 1 hour at room temperature. The conjugate solution was removed by aspiration and the plate was washed three times with PBS. A HRP substrate (Pierce ODP, made to the manufacturer's

instructions) was added (320 microliters) to each well and the colorimetric reaction proceeded for 30 minutes. The HRP reaction was stopped at 30 minutes by pipetting 50 microliters of 1.2N sulfuric acid into each well using a multi-channel pipetter. The absorbance at 490 nm was read in a microtiter plate reader. In order to compare results in separate experiments, data was normalized by expressing the signal generated in uteroglobin-fibronectin test wells as a percent of the signal from fibronectin alone.

Fibronectin is an >200 kDa glycoprotein with three types of repeating elements, called domains, which share a highly conserved secondary structure and a moderately conserved primary amino acid sequence. There are eight type I domains located in the N-terminal third of the human fibronectin protomer and three type I domains at the C-terminus of hFn. There are two type II domains, clustered in the middle of the protomer. There are between 15-17 type III domains in hFn, depending upon the tissue of origin of the fibronectin. In plasma fibronectin, the circulating type produced by Life Technologies Inc. the liver, Type III domains EDA and EDB are not present.

An N-terminal proteolytic fragment of hFn was not available and could not be tested. However, the 120 kDa and 40 kDa chymotryptic fragments of hFn (obtained from Life Technologies Inc.) encompass about 70% of the length of the intact fibronectin molecule, as shown in the map of fibronectin in figure 18. Type III domains #1-11 are present in the 120 kDa chymotryptic fragment as shown in the diagram. Type III domains #12-17 are present in the 40 kDa chymotryptic fragment. The clear dose-response curve for the 40 kDa chymotryptic fragment shows that recombinant human uteroglobin binds to at least one other hFn type III domain present in this fragment (#12-17). The recombinant type III domain #1 (referred to as III.1) is found within the 120 kDa chymotryptic fragment (see figure 18).

Reports that recombinant human uteroglobin and human fibronectin form a complex in solution with a measured binding constant of 13 nM and demonstrated biological activity *in vivo* in mice indicate that an uteroglobin -fibronectin complex could exist *in vivo* in humans. Indeed, high background readings for the purified human fibronectin using the anti-uteroglobin antibody (DAKO) in this uteroglobin-fibronectin binding assay led to speculation that there may be endogenous native uteroglobin that copurified with the fibronectin from human plasma. However, this solution phase interaction occurs at much lower concentrations of uteroglobin and Fn than the interaction seen between recombinant human uteroglobin and insoluble fibronectin. In addition, the solution phase interaction was shown to be relevant to fibronectin polymerization, conversion to

the insoluble form, the initial fibronectin deposition in the extracellular matrix or on cells, and in the process of fibrillogenesis. At low concentrations of plated fibronectin and recombinant human uteroglobin no interaction can be detected in this assay. On the basis of this apparent difference, it can be inferred that the downstream process of cell adhesion to the deposited fibronectin during inflammatory cell and fibroblast migration may also be effected by the presence of uteroglobin. Therefore, the following hypotheses were tested in the experiment below: (1) is there uteroglobin-like antigen in the intact hFn preparation, and (2) does recombinant human uteroglobin bind to portions of fibronectin that are important in cell adhesion and not relevant to fibrillogenesis.

Two commercially available chymotryptic fragments of hFn were selected for experimentation. These fragments were more highly purified than the hFn, having gone through several stages of chromatographic purification after proteolytic cleavage. These chymotryptic fragments are referred to as the 120 kDa fragment and the 40 kDa fragment, both type III domains that are involved in cell adhesion. However, only the 120 kDa fragment contains the region of fibronectin required for polymerization. Fibronectin self-polymerization activity has been localized to an N-terminal 70 kDa proteolytic fragment of human fibronectin, specifically a region including the type I.9-type III.1 domains. The C-terminal 70 amino acid part of the type III.1 domain was produced recombinantly (by Morla et al., 1994) and dubbed "Superfibronectin" because of its tremendous ability to promote fibronectin-fibronectin interactions, polymerization, deposition, and cell adhesion *in vitro*. Superfibronectin also has the advantage that no endogenous human uteroglobin could possibly be present in the protein preparation because it was purified from bacteria.

The three pieces of fibronectin were tested in parallel with the intact purified human fibronectin. Five micrograms per well of each Fn species were tested as described above, for binding to 250 nanograms of recombinant human uteroglobin/well. Two such experiments were performed, in duplicate, and the results are shown in figure 19.

In the absence of recombinant human uteroglobin the two chymotryptic fragments and the recombinant type III domain, did not show an appreciable signal. Therefore, endogenous uteroglobin is present in the purified intact fibronectin preparation at an approximate concentration of 250 nanograms of uteroglobin per 5 micrograms of hFn. This represents a molar ratio of 17:1, using molecular weights of 16 kDa for the uteroglobin homodimer and 200 kDa for the fibronectin protomer. Second, the binding of the recombinant human uteroglobin to both the 120 kDa and the

40 kDa fragments shows that there is more than one recombinant human uteroglobin binding site in plated fibronectin. The binding of recombinant human uteroglobin to superfibronectin localizes this multiple binding phenomena to the Fn type III repeats, which are present in both the 120 kDa and the 40 kDa fragments.

A titration of recombinant human uteroglobin was also done with each fibronectin preparation, in parallel. All four of these preparations were used to coat plates as described, using one microgram of protein per well. Titration curves were done in which various amounts of recombinant human uteroglobin were bound to the intact purified hFn, the two fragments and the recombinant superfibronectin as shown in figure 20. This experiment was done in duplicate and the uteroglobin-fibronectin binding step was done in the presence of 2.7 mM calcium chloride, in 1X PBS (pH 7.4) (which is consistent with the normal concentration of calcium in the serum).

There are three conclusions to be drawn from these results: first, the clear dose-response relationship in binding between recombinant human uteroglobin and superfibronectin shows that recombinant human uteroglobin binds to the domain of fibronectin which is also contained in superfibronectin; second, recombinant human uteroglobin binds to more than one Fn Type III domain, because there is a clear dose-response relationship for recombinant human uteroglobin binding to the 40 kDa chymotryptic fragment, which does not contain the superfibronectin peptide sequence, i.e., the type III.1 domain.

The Fn III.10 domain is known as the cell adhesion domain and it contains the only "RGDS" cell adhesion motif present in the entire hFn molecule. Mutagenesis of this sequence to "RGES" severely diminishes adhesion of fibroblasts and pro-inflammatory immune cells, such as neutrophils, to fibronectin. A monoclonal antibody against the cell adhesion domain, clone 3E3, is capable of blocking cell adhesion to fibronectin. Since recombinant human uteroglobin binds to the insoluble cell adhesion domain and to insoluble intact hFn and its chymotryptic fragments, it may be capable of blocking cell adhesion to fibronectin, via an anti-inflammatory, anti-fibrotic and anti-metastatic mechanism.

This hypothesis was tested in cell culture by assaying adhesion of NIH-3T3 cells (ATCC deposit # CRL-6589), an immortalized mouse fibroblast cell line, to hFn-coated wells (BioCoat plates, supplier). This cell line is a senescent diploid cell line, that more closely represents human lung fibroblasts *in vivo* than do numerous transformed and cancer cell lines available. The average

results from two of experiments are shown in the table below and clearly demonstrate that recombinant human uteroglobin is a potent inhibitor of cellular adhesion to fibronectin.

Table 9

	Recombinant human uteroglobin 50ng	Anti-Fn mAb 10µg	Myoglobin 10µg
% Inhibition	54%	60%	21%

Clearly, the recombinant human uteroglobin was a potent inhibitor of cellular adhesion to fibronectin. It was nearly as effective as the anti-Fn mAb, but at a 200-fold lower concentration. Myoglobin is a protein that is thought to be irrelevant to the physiology of both uteroglboin and fibronectin and was selected as a non-specific protein control because it is also a circulating protein that is roughly the same size as uteroglobin.

Both the uteroglobin 4 helix motif and the Fn Type III domain represent fundamental protein structural motifs present in many different proteins and it is reasonable to infer that these motifs share a certain affinity for each other, independent of the context of each individual protein. In fact, all 17 isolated hFn Type III domains mediate cell adhesion to some degree. It, therefore, follows that it is the availability of these domains for binding to other moieties in the surrounding environment that determines whether hFn interacts with other proteins, the extracellular matrix, or with cells. Indeed, the conformation of hFn is known to change from an elongated disk-like globular shape in solution to a stretched-out Y shape upon deposition onto a surface (Erickson & Carrell, 1983). The Fn Type III domains are present in nearly all protein components of the extracellular matrix (e.g., laminin, collagens, vitronectin, fibrin) as well as numerous membrane bound proteins, including adhesion molecules, integrins, and receptors. This 70 amino acid motif takes on a characteristic secondary structure that is thought to be absolutely conserved, despite differences in primary amino acid sequence.

Fibronectin Type III repeats are found in a large number of extracellular matrix proteins, as well as in a number of cell surface receptors. Based on their distribution in certain proteins, these domains would seem to play an integral role in cell-cell and cell-extracellular matrix interactions.

Example 3

The discovery that recombinant human uteroglobin binds to human fibronectin in solution has profound implications (USSN 08/857,364). In addition, the ability of recombinant human uteroglobin to prevent fibronectin aggregation *in vitro*, fibronectin-mediated fibrillogenesis in cell culture, and renal fibronectin deposition *in vivo*, demonstrates the important physiological role of endogenous uteroglobin in all mammals. Fibronectin is one of the most well characterized mediators of cell adhesion, and is involved in several physiologic processes, including platelet aggregation (thrombosis), wound healing, fibrosis, inflammatory cell and fibroblast adhesion, tumor metastases, and extracellular basement membrane formation. However, these processes involve the insoluble form of fibronectin, not the soluble form. It would be desirable to prevent the conversion of fibronectin from its soluble form to its insoluble form, which could be exploited to prevent the initiation of the processes listed above, or in limiting the extent of such processes. It has now been found that recombinant human uteroglobin also binds to insoluble fibronectin.

When fibronectin converts to its insoluble form, it changes conformation and deposits on the surfaces of cells and on the extracellular matrix, where it may act as an anchor for polymorphonuclear leukocytes, macrophages, monocytes, and fibroblasts during an inflammatory episode. The receptors for fibronectin on cell surfaces are numerous and fall into different classes of molecules, including cell adhesion molecules (ie. ICAM-1) and integrin complexes (ie. a1b4 integrin). These types of molecules mediate not only cell adhesion but also provide a means through which the cell senses, and reacts to, its environment. Recombinant human uteroglobin specifically binds to fragments of fibronectin that contain type III domains, and the RGD-containing type III domain (#10), in particular. The RGD peptide is well known as a mediator of cell adhesion in leukocyte extravasation during inflammation. RGD-containing peptides are potent inhibitors of cell adhesion *in vitro*, for many types of mammalian cells. Commercial interests have attempted to use RGD peptides and peptidomimetics as anti-inflammatory agents *in vivo*, but have met with limited success due to the instability of these types of compounds. Therefore, the potential use of recombinant human uteroglobin as an inhibitor of cell adhesion to fibronectin *in vitro* was investigated.

Cellular adhesion assays were performed essentially as described by Retta, et al. ("Adhesion to Matrix Proteins" in Methods in Molecular Biology, Dejana, E. and M. Corada, Eds., 96: 125-130, Humana Press, Totowa, NJ; 1999). Briefly, a 96-well fibronectin-coated plate was blocked (to

prevent non-specific binding of cells or proteins to the plastic) with 1X Pierce Blocker™, containing 5% BSA, at 37°C in incubator for at least 1 hour. The blocking reagent was then removed and the wells were washed three times with PBS (phosphate buffered saline). The prepared plate were stored in the incubator while preparing the cells. Two cell lines, NIH 3T3 and Hela cells (ATCC # CCL-2) were selected for the assay. NIH 3T3 cells have been shown to have a high density of the uteroglobin receptor and Hela cells do not have the uteroglobin receptor. Both lines were grown with standard tissue culture techniques in Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum. The cells were removed from culture flasks using 5 mM EDTA and spun down gently. The cells were then washed three times by alternately resuspending in serum-free medium and repeat centrifugation. The final cell pellet was resuspended to an estimated density of 2.5 - 7.5 x 10^4 cells per 200 ml. Cells were partitioned into aliquots to receive either uteroglobin, a monoclonal antibody control, Type 1b secretory PLA2 (porcine pancreatic) ($ppPLA_2$), myoglobin (dog heart) control, or PBS control. Because uteroglobin and $sPLA_2$s share structural similarity, PLA_2 was tested as well.

Cells were then added to each well of the fibronectin-coated plate as quickly as possible. The plates were incubated at 37°C in a CO2 incubator for 1 hour. While the plated cells were incubating, live cells in the pipetted suspension were counted by trypan blue dye exclusion using a hemacytometer. At one hour the wells of the plate were aspirated and washed one time with PBS to remove non-adhered cells. The adhered cells were then fixed to the plate with 3.7% paraformaldehyde in PBS for 10^1 at room temperature. The plates were then washed once with PBS and the adherent cells were stained by adding 200 ml of 0.25% R250 Coomassie blue to each well and allowing the cells to stand at room temp. for 1 hour. The stain solution was then aspirated and wells washed three times with PBS. Adherent cells were quantitated by reading the optical density at 540 nm in microtiter plate reader.

The tabulated resultss shown below are reported as the percent inhibition of cellular adhesion. Percent inhibition was calculated as 100% minus the ratio of the mean OD of the test protein over the mean OD of the PBS alone (no protein) control. All protein groups were run in triplicate in each experiment. All numbers represent the mean percent inhibition for three separate experiments.

Table 10A - Effects of Uteroglobin on Cellular Adhesion to Fibronectin; NIH-3T3 Cell line (high density of uteroglobin receptor(s); mouse lung fibroblasts)

	Uteroglobin (50 ng)	Anti-fibronectin monoclonal antibody (10 μg)	$ppPLA_2$ (50 ng)	Control (10 μg)
Percent Inhibition	54%	60%	68%	31%

Table 10B - Effects of Uteroglobin on Cellular Adhesion to Fibronectin; Hela Cell line (low density of uteroglobin receptor(s); human cervical carcinoma)

	Uteroglobin (50 ng)	Anti-fibronectin monoclonal antibody (10 μg)	$ppPLA_2$ (50 ng)	Control (50 ng)
Percent Inhibition	-5%	ND	28.5%	23%

The above description of the invention is intended to be illustrative and not limiting. Various changes or modifications in the embodiments described may occur to those skilled in the art. These can be made without departing from the spirit or scope of the invention.

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